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RNA Interference Mediating Small RNA molecules

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**RNA Interference Mediating Small RNA molecules**EPO - Munich  
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**Description**

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The present invention relates to sequence and structural features of double-stranded (ds)RNA molecules required to mediate target-specific nucleic acid modifications such as RNA-interference and/or DNA methylation.

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The term "RNA interference" (RNAi) was coined after the discovery that injection of dsRNA into the nematode *C. elegans* leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA (Fire et al., 1998). RNAi was subsequently also observed in insects, frogs (Oelgeschlager et al., 2000), and other animals including mice (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000) and is likely to also exist in human. RNAi is closely linked to the post-transcriptional gene-silencing (PTGS) mechanism of co-suppression in plants and quelling in fungi (Catalanotto et al., 2000; Cogoni and Macino, 1999; Dalmay et al., 2000; Ketting and Plasterk, 2000; Mourrain et al., 2000; Smardon et al., 2000) and some components of the RNAi machinery are also necessary for post-transcriptional silencing by co-suppression (Catalanotto et al., 2000; Dernburg et al., 2000; Ketting and Plasterk, 2000). The topic has also been reviewed recently (Bass, 2000; Boshier and Labouesse, 2000; Fire, 1999; Plasterk and Ketting, 2000; Sharp, 1999; Sijen and Kooter, 2000), see also the entire issue of *Plant Molecular Biology*, vol. 43, issue 2/3, (2000).

In plants, in addition to PTGS, introduced transgenes can also lead to transcriptional gene silencing via RNA-directed DNA methylation of cytosines (see references in Wassenegger, 2000). Genomic targets as short as 30 bp are methylated in plants in an RNA-directed manner (Pelissier, 2000). DNA methylation is also present in mammals.

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The natural function of RNAi and co-suppression appears to be protection of the genome against invasion by mobile genetic elements such as retro-transposons and viruses which produce aberrant RNA or dsRNA in the host cell when they become active (Jensen et al., 1999; Ketting et al., 1999; Ratcliff et al., 1999; Tabara et al., 1999). Specific mRNA degradation prevents transposon and virus replication although some viruses are able to overcome or prevent this process by expressing proteins that suppress PTGS (Lucy et al., 2000; Voinnet et al., 2000).

DsRNA triggers the specific degradation of homologous RNAs only within the region of identity with the dsRNA (Zamore et al., 2000). The dsRNA is processed to 21-23 nt RNA fragments and the target RNA cleavage sites are regularly spaced 21-23 nt apart. It has therefore been suggested that the 21-23 nt fragments are the guide RNAs for target recognition (Zamore et al., 2000). These short RNAs were also detected in extracts prepared from *D. melanogaster* Schneider 2 cells which were transfected with dsRNA prior to cell lysis (Hammond et al., 2000), however, the fractions that displayed sequence-specific nuclease activity also contained a large fraction of residual dsRNA. The role of the 21-23 nt fragments in guiding mRNA cleavage is further supported by the observation that 21-23 nt fragments isolated from processed dsRNA are able, to some extent, to mediate specific mRNA degradation (Zamore et al., 2000). RNA molecules of similar size also accumulate in plant tissue that exhibits PTGS (Hamilton and Baulcombe, 1999).

Here, we use the established *Drosophila* in vitro system (Tuschl et al., 1999; Zamore et al., 2000) to further explore the mechanism of RNAi. We demonstrate that short 21 and 22 nt RNAs, when base-paired with 3' overhanging ends, act as the guide RNAs for sequence-specific mRNA degradation. Short 30 bp dsRNAs are unable to mediate RNAi because they are no longer processed to 21 and 22 nt RNAs. Furthermore, we defined the target RNA cleavage sites relative to the 21 and 22 nt short

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interfering RNAs (siRNAs) and provide evidence that the direction of dsRNA processing determines whether a sense or an antisense target RNA can be cleaved by the produced siRNP endonuclease complex. Further, the siRNAs may also be important tools for transcriptional modulating, e.g. silencing of mammalian genes by guiding DNA methylation.

The object underlying the present invention is to provide novel agents capable of mediating target-specific RNA interference or other target-specific nucleic acid modifications such as DNA methylation, said agents having an improved efficacy and safety compared to prior art agents.

The solution of this problem is provided by an isolated double-stranded RNA molecule, wherein each RNA strand has a length from 19-23 nucleotides, wherein said RNA molecule is capable of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation. Preferably at least one strand has a 3'-overhang from 1-5 nucleotides. The other strand may be blunt-ended or has up to 6 nucleotides 3' overhang. Also, if both strands of the dsRNA are exactly 21 or 22 nt, it is possible to observe some RNA interference when both ends are blunt (0 nt overhang). The RNA molecule is preferably a synthetic RNA molecule which is substantially free from contaminants occurring in cell extracts, e.g. from *Drosophila* embryos. Further, the RNA molecule is preferably substantially free from any non-target-specific contaminants, particularly non-target-specific RNA molecules e.g. from contaminants occurring in cell extracts.

Surprisingly, it was found that synthetic short double-stranded RNA molecules particularly with overhanging 3'-ends are sequence-specific mediators of RNAi and mediate efficient target-RNA cleavage, wherein the cleavage site is located near the center of the region spanned by the guiding short RNA.

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Preferably, each strand of the RNA molecule has a length from 20-22 nucleotides, wherein the length of each strand may be the same or different. Preferably, the length of the 3'-overhang reaches from 1-3 nucleotides, wherein the length of the overhang may be the same or different for each strand. The RNA-strands preferably have 3'-hydroxyl groups. The 5'-terminus preferably comprises a phosphate, diphosphate, triphosphate or hydroxyl group. The most effective dsRNAs are composed of two 21 nt strands which are paired such that 1-3, particularly 2 nt 3' overhangs are present on both ends of the dsRNA. Pyrimidine nucleotides in the 3' overhang are more effective in mediating RNA interference than purine nucleotides. Also, the nucleotides present in the 3' overhang do not seem to contribute to the specificity of target RNA recognition.

Surprisingly, the double-stranded RNA molecules of the present invention exhibit a high in vivo stability in serum or in growth medium for cell cultures. In order to further enhance the stability, the 3'-overhangs may be stabilized against degradation, e.g. they may be selected such that they consist of purine nucleotides, particularly adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g. Substitution of uridine 2 nt 3' overhangs by 2'-deoxythymidine is tolerated and does not affect the efficiency of RNA interference. The absence of a 2' hydroxyl significantly enhances the nuclease resistance of the overhang in tissue culture medium.

In an especially preferred embodiment of the present invention the RNA molecule may contain at least one modified nucleotide analogue. The nucleotide analogues may be located at positions where the target-specific activity, e.g. the RNAi mediating activity is not substantially effected, e.g. in a region at the 5'-end and/or the 3'-end of the double-stranded RNA molecule. Particularly, the overhangs may be stabilized by incorporating modified nucleotide analogues.

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Preferred nucleotide analogues are selected from sugar- or backbone-modified ribonucleotides. It should be noted, however, that also nucleobase-modified ribonucleotides, i.e. ribonucleotides, containing a non-naturally occurring nucleobase instead of a naturally occurring nucleobase such as uridines or cytidines modified at the 5-position, e.g. 5-(2-amino)propyl uridine, 5-bromo uridine; adenosines and guanosines modified at the 8-position, e.g. 8-bromo guanosine; deaza nucleotides, e.g. 7-deaza-adenosine; O- and N-alkylated nucleotides, e.g. N6-methyl adenosine are suitable. In preferred sugar-modified ribonucleotides the 2' OH-group is replaced by a group selected from H, OR, R, halo, SH, SR, NH<sub>2</sub>, NHR, NR<sub>2</sub> or CN, wherein R is C<sub>1</sub>-C<sub>6</sub> alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I. In preferred backbone-modified ribonucleotides the phosphoester group connecting to adjacent ribonucleotides is replaced by a modified group, e.g. of phosphothioate group. It should be noted that the above modifications may be combined.

The sequence of the double-stranded RNA molecule of the present invention has to have a sufficient identity to a nucleic acid target molecule in order to mediate target-specific RNAi and/or DNA methylation. Preferably, the sequence has an identity of at least 50%, particularly of at least 70% to the desired target molecule in the double-stranded portion of the RNA molecule. More preferably, the identity is at least 70% and most preferably 100% in the double-stranded portion of the RNA molecule. The identity of a double-stranded RNA molecule to a predetermined nucleic acid target molecule, e.g. an mRNA target molecule may be determined as follows:

$$I = \frac{n}{L} \times 100$$

wherein I is the identity in percent, n is the number of identical nucleotides in the double-stranded portion of the ds RNA and the target and L is the length of the sequence overlap of the double-stranded portion of the dsRNA and the target.



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The double-stranded RNA molecule of the invention may be prepared by a method comprising the steps:

- 5 (a) synthesizing two RNA strands each having a length from 19-23 nucleotides, wherein said RNA strands are capable of forming a double-stranded RNA molecule, wherein preferably at least one strand has a 3'-overhang from 1-5 nucleotides,
- 10 (b) combining the synthesized RNA strands under conditions, wherein a double-stranded RNA molecule is formed, which is capable of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation.

15 Methods of synthesizing RNA molecules are known in the art. In this context, it is particularly referred to chemical synthesis methods as described in Verma and Eckstein (1998).

The single-stranded RNAs can also be prepared by enzymatic transcription from synthetic DNA templates or from DNA plasmids isolated from recombinant bacteria. Typically, phage RNA polymerases are used such as T7, T3 or SP6 RNA polymerase (Milligan and Uhlenbeck (1989)).

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A further aspect of the present invention relates to a method of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation in a cell or an organism comprising the steps:

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- (a) contacting the cell or organism with the double-stranded RNA molecule of the invention and
- 30 (b) mediating a target-specific nucleic acid modification effected by the double-stranded RNA towards a target nucleic acid having a

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sequence portion substantially corresponding to the double-stranded RNA.

The method of the invention may be used for determining the function of a gene in a cell or an organism or even for modulating the function of a gene in a cell or an organism, being capable of mediating RNA interference. The cell is preferably a eukaryotic cell or a cell line, e.g. a plant cell or an animal cell, such as a mammalian cell, e.g. an embryonic cell, a pluripotent stem cell, a tumor cell, e.g. a teratocarcinoma cell or a virus-infected cell. The organism is preferably a eukaryotic organism, e.g. a plant or an animal, such as a mammal, particularly a human.

The target gene to which the RNA molecule of the invention is directed may be associated with a pathological condition. For example, the gene may be a pathogen-associated gene, e.g. a viral gene, a tumor-associated gene or an autoimmune disease-associated gene. The target gene may also be a heterologous gene expressed in a recombinant cell or a genetically altered organism. By determinating or modulating, particularly, inhibiting the function of such a gene valuable information and therapeutic benefits in the agricultural field or in the medicine or veterinary medicine field may be obtained.

The dsRNA is usually administered as a pharmaceutical composition. The administration may be carried out by known methods, wherein a nucleic acid is introduced into a desired target cell in vitro or in vivo. Commonly used gene transfer techniques include calcium phosphate, DEAE-dextran, electroporation and microinjection and viral methods (Graham, F.L. and van der Eb, A.J. (1973) Virol. 52, 456; McCutchan, J.H. and Pagano, J.S. (1968), J. Natl. Cancer Inst. 41, 351; Chu, G. et al (1987), Nucl. Acids Res. 15, 1311; Fraley, R. et al. (1980), J. Biol. Chem. 255, 10431; Capecchi, M.R. (1980), Cell 22, 479). A recent addition to this arsenal of techniques for the introduction of DNA into cells is the use of cationic liposomes

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(Felgner, P.L. et al. (1987), Proc. Natl. Acad. Sci USA 84, 7413). Commercially available cationic lipid formulations are e.g. Tfx 50 (Promega) or Lipofectamin2000 (Life Technologies).

5 Thus, the invention also relates to a pharmaceutical composition containing as an active agent at least one double-stranded RNA molecule as described above and a pharmaceutical carrier. The composition may be used for diagnostic and for therapeutic applications.

10 For diagnostic or therapeutic applications, the composition may be in form of a solution, e.g. an injectable solution, a cream, ointment, tablet, suspension or the like. The composition may be administered in any suitable way, e.g. by injection, by oral, topical, nasal, rectal application etc. The carrier may be any suitable pharmaceutical carrier. Preferably, a carrier is used,  
15 which is capable of increasing the efficacy of the RNA molecules to enter the target-cells. Suitable examples of such carriers are liposomes, particularly cationic liposomes.

Further, the present invention is explained in more detail in the following  
20 figures and examples.

### Figure Legends

**Figure 1:** Double-stranded RNA as short as 38 bp can mediate RNAi.

25 (A) Graphic representation of dsRNAs used for targeting Pp-luc mRNA. Three series of blunt-ended dsRNAs covering a range of 29 to 504 bp were prepared. The position of the first nucleotide of the sense strand of the dsRNA is indicated relative to the start codon of Pp-luc mRNA (p1). (B)  
RNA interference assay (Tuschl et al., 1999) . Ratios of target Pp-luc to  
30 control Rr-luc activity were normalized to a buffer control (black bar). DsRNAs (5 nM) were preincubated in Drosophila lysate for 15 min at 25°C prior to the addition of 7-methyl-guanosine-capped Pp-luc and Rr-luc

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mRNAs ( $\sim 50$  pM). The incubation was continued for another hour and then analyzed by the dual luciferase assay (Promega). The data are the average from at least four independent experiments  $\pm$  standard deviation.

5 **Figure 2:** A 29 bp dsRNA is no longer processed to 21-23 nt fragments. Time course of 21-23 mer formation from processing of internally  $^{32}\text{P}$ -labeled dsRNAs (5 nM) in the *Drosophila* lysate. The length and source of the dsRNA are indicated. An RNA size marker (M) has been loaded in the left lane and the fragment sizes are indicated. Double bands at time zero  
10 are due to incompletely denatured dsRNA.

**Figure 3:** Short dsRNAs cleave the mRNA target only once.

(A) Denaturing gel electrophoreses of the stable 5' cleavage products produced by 1 h incubation of 10 nM sense or antisense RNA  $^{32}\text{P}$ -labeled  
15 at the cap with 10 nM dsRNAs of the p133 series in *Drosophila* lysate. Length markers were generated by partial nuclease T1 digestion and partial alkaline hydrolysis (OH) of the cap-labeled target RNA. The regions targeted by the dsRNAs are indicated as black bars on both sides. The 20-23 nt spacing between the predominant cleavage sites for the 111 bp long  
20 dsRNA is shown. The horizontal arrow indicates unspecific cleavage not due to RNAi. (B) Position of the cleavage sites on sense and antisense target RNAs. The sequences of the capped 177 nt sense and 180 nt antisense target RNAs are represented in antiparallel orientation such that complementary sequence are opposing each other. The region targeted by  
25 the different dsRNAs are indicated by differently colored bars positioned between sense and antisense target sequences. Cleavage sites are indicated by circles: large circle for strong cleavage, small circle for weak cleavage. The  $^{32}\text{P}$ -radiolabeled phosphate group is marked by an asterisk.

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**Figure 4:** 21 and 22 nt RNA fragments are generated by an RNase III-like mechanism.

(A) Sequences of ~21 nt RNAs after dsRNA processing. The ~21 nt RNA fragments generated by dsRNA processing were directionally cloned and sequenced. Oligoribonucleotides originating from the sense strand of the dsRNA are indicated as blue lines, those originating from the antisense strand as red lines. Thick bars are used if the same sequence was present in multiple clones, the number at the right indicating the frequency. The target RNA cleavage sites mediated by the dsRNA are indicated as orange circles, large circle for strong cleavage, small circle for weak cleavage (see Figure 3B). Circles on top of the sense strand indicated cleavage sites within the sense target and circles at the bottom of the dsRNA indicate cleavage site in the antisense target. Up to five additional nucleotides were identified in ~21 nt fragments derived from the 3' ends of the dsRNA. These nucleotides are random combinations of predominantly C, G, or A residues and were most likely added in an untemplated fashion during T7 transcription of the dsRNA-constituting strands. (B) Two-dimensional TLC analysis of the nucleotide composition of ~21 nt RNAs. The ~21 nt RNAs were generated by incubation of internally radiolabeled 504 bp Pp-luc dsRNA in *Drosophila* lysate, gel-purified, and then digested to mononucleotides with nuclease P1 (top row) or ribonuclease T2 (bottom row). The dsRNA was internally radiolabeled by transcription in the presence of one of the indicated  $\alpha$ -<sup>32</sup>P nucleoside triphosphates. Radioactivity was detected by phosphorimaging. Nucleoside 5'-monophosphates, nucleoside 3'-monophosphates, nucleoside 5',3'-diphosphates, and inorganic phosphate are indicated as pN, Np, pNp, and p<sub>i</sub>, respectively. Black circles indicate UV-absorbing spots from non-radioactive carrier nucleotides. The 3',5'-bisphosphates (red circles) were identified by co-migration with radiolabeled standards prepared by 5'-phosphorylation of nucleoside 3'-monophosphates with T4 polynucleotide kinase and  $\gamma$ -<sup>32</sup>P-ATP.

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**Figure 5: Synthetic 21 and 22 nt RNAs Mediate Target RNA Cleavage**

(A) Graphic representation of control 52 bp dsRNA and synthetic 21 and 22 nt dsRNAs. The sense strand of 21 and 22 nt short interfering RNAs (siRNAs) is shown blue, the antisense strand in red. The sequences of the siRNAs were derived from the cloned fragments of 52 and 111 bp dsRNAs (Figure 4A), except for the 22 nt antisense strand of duplex 5. The siRNAs in duplex 6 and 7 were unique to the 111 bp dsRNA processing reaction. The two 3' overhanging nucleotides indicated in green are present in the sequence of the synthetic antisense strand of duplexes 1 and 3. Both strands of the control 52 bp dsRNA were prepared by in vitro transcription and a fraction of transcripts may contain untemplated 3' nucleotide addition. The target RNA cleavage sites directed by the siRNA duplexes are indicated as orange circles (see legend to Figure 4A) and were determined as shown in Figure 5B. (B) Position of the cleavage sites on sense and antisense target RNAs. The target RNA sequences are as described in Figure 3B. Control 52 bp dsRNA (10 nM) or 21 and 22 nt RNA duplexes 1-7 (100 nM) were incubated with target RNA for 2.5 h at 25°C in *Drosophila* lysate. The stable 5' cleavage products were resolved on the gel. The cleavage sites are indicated in Figure 5A. The region targeted by the 52 bp dsRNA or the sense (s) or antisense (as) strands are indicated by the black bars to the side of the gel. The cleavage sites are all located within the region of identity of the dsRNAs. For precise determination of the cleavage sites of the antisense strand, a lower percentage gel was used.

**Figure 6: Long 3' overhangs on short dsRNAs inhibit RNAi**

(A) Graphic representation of 52 bp dsRNA constructs. The 3' extensions of sense and antisense strand are indicated in blue and red, respectively. The observed cleavage sites on the target RNAs are represented as orange circles analogous to Figure 4A and were determined as shown in Figure 6B. (B) Position of the cleavage sites on sense and antisense target RNAs. The target RNA sequences are as described in Figure 3B. DsRNA (10 nM) was incubated with target RNA for 2.5 h at 25°C in *Drosophila* lysate. The

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stable 5' cleavage products were resolved on the gel. The major cleavage sites are indicated with a horizontal arrow and also represented in Figure 6A. The region targeted by the 52 bp dsRNA is represented as black bar at both sides of the gel.

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#### Figure 7: Proposed Model for RNAi

RNAi is predicted to begin with processing of dsRNA (sense strand in black, antisense strand in red) to predominantly 21 and 22 nt short interfering RNAs (siRNAs). Short overhanging 3' nucleotides, if present on the dsRNA, may be beneficial for processing of short dsRNAs. The dsRNA-processing proteins, which remain to be characterized, are represented as green and blue ovals, and assembled on the dsRNA in asymmetric fashion. In our model, this is illustrated by binding of a hypothetical blue protein or protein domain with the siRNA strand in 3' to 5' direction while the hypothetical green protein or protein domain is always bound to the opposing siRNA strand. These proteins or a subset remain associated with the siRNA duplex and preserve its orientation as determined by the direction of the dsRNA processing reaction. Only the siRNA sequence associated with the blue protein is able to guide target RNA cleavage. The endonuclease complex is referred to as small interfering ribonucleoprotein complex or siRNP. It is presumed here, that the endonuclease that cleaves the dsRNA may also cleave the target RNA, probably by temporarily displacing the passive siRNA strand not used for target recognition. The target RNA is then cleaved in the center of the region recognized by the sequence-complementary guide siRNA.

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## Example

### 1. Experimental Procedures

#### 5 1.1 In Vitro RNAi

In vitro RNAi and lysate preparations were performed as described previously (Tuschl et al., 1999; Zamore et al., 2000). It is critical to use freshly dissolved creatine kinase (Roche) for optimal ATP regeneration. The RNAi translation assays (Fig. 1) were performed with dsRNA concentrations of 5 nM and an extended pre-incubation period of 15 min at 25°C prior to the addition of in vitro transcribed, capped and polyadenylated Pp-luc and Rr-luc reporter mRNAs. The incubation was continued for 1 h and the relative amount of Pp-luc and Rr-luc protein was analyzed using the dual luciferase assay (Promega) and a Monolight 3010C luminometer (PharMingen).

#### 1.2 RNA Synthesis

Standard procedures were used for in vitro transcription of RNA from PCR templates carrying T7 or SP6 promoter sequences, see for example (Tuschl et al., 1998). Synthetic RNA was prepared using Expedite RNA phosphoramidites (Proligo). The 3' adapter oligonucleotide was synthesized using dimethoxytrityl-1,4-benzenedimethanol-succinyl-aminopropyl-CPG. The oligoribonucleotides were deprotected in 3 ml of 32% ammonia/ethanol (3/1) for 4 h at 55°C (Expedite RNA) or 16 h at 55°C (3' and 5' adapter DNA/RNA chimeric oligonucleotides) and then desilylated and gel-purified as described previously (Tuschl et al., 1993). RNA transcripts for dsRNA preparation including long 3' overhangs were generated from PCR templates that contained a T7 promoter in sense and an SP6 promoter in antisense direction. The transcription template for sense and antisense target RNA was PCR-amplified with GCGTAATACGACTCACTATAGAAACAATTGCTTTTACAG (underlined, T7 promoter) as 5' primer and



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ATTTAGGTGACACTATAGGCATAAAGAATTGAAGA (underlined, SP6 promoter) as 3' primer and the linearized Pp-luc plasmid (pGEM-luc sequence) (Tuschl et al., 1999) as template; the T7-transcribed sense RNA was 177 nt long with the Pp-luc sequence between pos. 113-273 relative to the start codon and followed by 17 nt of the complement of the SP6 promoter sequence at the 3' end. Transcripts for blunt-ended dsRNA formation were prepared by transcription from two different PCR products which only contained a single promoter sequence.

DsRNA annealing was carried out using a phenol/chloroform extraction. Equimolar concentration of sense and antisense RNA (50 nM to 10  $\mu$ M, depending on the length and amount available) in 0.3 M NaOAc (pH 6) were incubated for 30 s at 90°C and then extracted at room temperature with an equal volume of phenol/chloroform, and followed by a chloroform extraction to remove residual phenol. The resulting dsRNA was precipitated by addition of 2.5 -3 volumes of ethanol. The pellet was dissolved in lysis buffer (100 mM KCl, 30 mM HEPES-KOH, pH 7.4, 2 mM Mg(OAc)<sub>2</sub>) and the quality of the dsRNA was verified by standard agarose gel electrophoreses in 1x TAE-buffer. The 52 bp dsRNAs with the 17 nt and 20 nt 3' overhangs (Figure 6) were annealed by incubating for 1 min at 95 °C, then rapidly cooled to 70°C and followed by slow cooling to room temperature over a 3 h period (50  $\mu$ l annealing reaction, 1  $\mu$ M strand concentration, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5). The dsRNAs were then phenol/chloroform extracted, ethanol-precipitated and dissolved in lysis buffer.

Transcription of internally <sup>32</sup>P-radiolabeled RNA used for dsRNA preparation (Figures 2 and 4) was performed using 1 mM ATP, CTP, GTP, 0.1 or 0.2 mM UTP, and 0.2-0.3  $\mu$ M <sup>32</sup>P-UTP (3000 Ci/mmol), or the respective ratio for radiolabeled nucleoside triphosphates other than UTP. Labeling of the cap of the target RNAs was performed as described previously. The target RNAs were gel-purified after cap-labeling.

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### 1.3 Cleavage Site Mapping

Standard RNAi reactions were performed by pre-incubating 10 nM dsRNA for 15 min followed by addition of 10 nM cap-labeled target RNA. The reaction was stopped after a further 2 h (Figure 2A) or 2.5 h incubation (Figure 5B and 6B) by proteinase K treatment (Tuschl et al., 1999). The samples were then analyzed on 8 or 10% sequencing gels. The 21 and 22 nt synthetic RNA duplexes were used at 100 nM final concentration (Fig 5B).

### 1.4 Cloning of ~21 nt RNAs

The 21 nt RNAs were produced by incubation of radiolabeled dsRNA in *Drosophila* lysate in absence of target RNA (200  $\mu$ l reaction, 1 h incubation, 50 nM dsP111, or 100 nM dsP52 or dsP39). The reaction mixture was subsequently treated with proteinase K (Tuschl et al., 1999) and the dsRNA-processing products were separated on a denaturing 15% polyacrylamide gel. A band, including a size range of at least 18 to 24 nt, was excised, eluted into 0.3 M NaCl overnight at 4°C and in siliconized tubes. The RNA was recovered by ethanol-precipitation and dephosphorylated (30  $\mu$ l reaction, 30 min, 50°C, 10 U alkaline phosphatase, Roche). The reaction was stopped by phenol/chloroform extraction and the RNA was ethanol-precipitated. The 3' adapter oligonucleotide (pUUUaaccgcatccttctcx: uppercase, RNA; lowercase, DNA; p, phosphate; x, 4-hydroxymethylbenzyl) was then ligated to the dephosphorylated ~21 nt RNA (20  $\mu$ l reaction, 30 min, 37°C, 5  $\mu$ M 3' adapter, 50 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 0.2 mM ATP, 0.1 mg/ml acetylated BSA, 15% DMSO, 25 U T4 RNA ligase, Amersham-Pharmacia) (Pan and Uhlenbeck, 1992). The ligation reaction was stopped by the addition of an equal volume of 8 M urea/50 mM EDTA stopmix and directly loaded on a 15% gel. Ligation yields were greater 50%. The ligation product was recovered from the gel and 5'-phosphorylated (20  $\mu$ l reaction, 30 min, 37°C, 2 mM ATP, 5 U T4 polynucleotide kinase, NEB). The phosphorylation reaction was stopped by phenol/chloroform extraction and RNA was recovered by ethanol-precipitation. Next, the

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5' adapter (tactaatagactcactAAA: uppercase, RNA; lowercase, DNA) was ligated to the phosphorylated ligation product as described above. The new ligation product was gel-purified and eluted from the gel slice in the presence of reverse transcription primer (GACTAGCTGGAATTCAAGGATGCGGTAAA: bold, Eco RI site) used as carrier. Reverse transcription (15  $\mu$ l reaction, 30 min, 42°C, 150 U Superscript II reverse transcriptase, Life Technologies) was followed by PCR using as 5' primer CAGCCAACGGAATTCATACGACTCACTAAA (bold, Eco RI site) and the 3' RT primer. The PCR product was purified by phenol/chloroform extraction and ethanol-precipitated. The PCR product was then digested with Eco RI (NEB) and concatamerized using T4 DNA ligase (high conc., NEB). Concatamers of a size range of 200 to 800 bp were separated on a low-melt agarose gel, recovered from the gel by a standard melting and phenol extraction procedure, and ethanol-precipitated. The unpaired ends were filled in by incubation with Taq polymerase under standard conditions for 15 min at 72°C and the DNA product was directly ligated into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen). Colonies were screened using PCR and M13-20 and M13 Reverse sequencing primers. PCR products were directly submitted for custom sequencing (Sequence Laboratories Göttingen GmbH, Germany). On average, four to five 21mer sequences were obtained per clone.

### 1.5 2D-TLC Analysis

Nuclease P1 digestion of radiolabeled, gel-purified siRNAs and 2D-TLC was carried out as described (Zamore et al., 2000). Nuclease T2 digestion was performed in 10  $\mu$ l reactions for 3 h at 50°C in 10 mM ammonium acetate (pH 4.5) using 2  $\mu$ g/ $\mu$ l carrier tRNA and 30 U ribonuclease T2 (Life Technologies). The migration of non-radioactive standards was determined by UV shadowing. The identity of nucleoside-3',5'-disphosphates was confirmed by co-migration of the T2 digestion products with standards prepared by 5'-<sup>32</sup>P-phosphorylation of commercial nucleoside 3'-monophosphates using  $\gamma$ -<sup>32</sup>P-ATP and T4 polynucleotide kinase (data not shown).

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## 2. Results and Discussion

### 2.1 Length Requirements for Processing of dsRNA to 21 and 22 nt RNA Fragments

5 Lysate prepared from *D. melanogaster* syncytial embryos recapitulates RNAi in vitro providing a novel tool for biochemical analysis of the mechanism of RNAi (Tuschl et al., 1999; Zamore et al., 2000). In vitro and in vivo analysis of the length requirements of dsRNA for RNAi has revealed that short dsRNA (<150 bp) are less effective than longer dsRNAs in  
10 degrading target mRNA (Caplen et al., 2000; Hammond et al., 2000; Ngo et al., 1998); Tuschl et al., 1999). The reasons for reduction in mRNA degrading efficiency are not understood. We therefore examined the precise length requirement of dsRNA for target RNA degradation under optimized conditions in the *Drosophila* lysate (Zamore et al., 2000). Several  
15 series of dsRNAs were synthesized and directed against firefly luciferase (Pp-luc) reporter RNA. The specific suppression of target RNA expression was monitored by the dual luciferase assay (Tuschl et al., 1999) (Figures 1A and 1B). We detected specific inhibition of target RNA expression for dsRNAs as short as 38 bp, but dsRNAs of 29 to 36 bp were not effective  
20 in this process. The effect was independent of the target position and the degree of inhibition of Pp-luc mRNA expression correlated with the length of the dsRNA, i.e. long dsRNAs were more effective than short dsRNAs.

It has been suggested that the 21-23 nt RNA fragments generated by  
25 processing of dsRNAs are the mediators of RNA interference and co-suppression (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000). We therefore analyzed the rate of 21-23 nt fragment formation for a subset of dsRNAs ranging in size between 501 to 29 bp. Formation of 21-23 nt fragments in *Drosophila* lysate (Figure 2) was readily  
30 detectable for 39 to 501 bp long dsRNAs but was significantly delayed for the 29 bp dsRNA. This observation is consistent with a role of 21-23 nt fragments in guiding mRNA cleavage and provides an explanation for the

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lack of RNAi by 30 bp dsRNAs. The length dependence of 21-23 mer formation is likely to reflect a biologically relevant control mechanism to prevent the undesired activation of RNAi by short intramolecular base-paired structures of regular cellular RNAs.

5

## 2.2 39 bp dsRNA Mediates Target RNA Cleavage at a Single Site

Addition of dsRNA and 5'-capped target RNA to the *Drosophila* lysate results in sequence-specific degradation of the target RNA (Tuschl et al., 1999). The target mRNA is only cleaved within the region of identity with the dsRNA and many of the target cleavage sites were separated by 21-23 nt (Zamore et al., 2000). Thus, the number of cleavage sites for a given dsRNA was expected to roughly correspond to the length of the dsRNA divided by 21. We mapped the target cleavage sites on a sense and an antisense target RNA which was 5' radiolabeled at the cap (Zamore et al., 2000) (Figures 3A and 3B). Stable 5' cleavage products were separated on a sequencing gel and the position of cleavage was determined by comparison with a partial RNase T1 and an alkaline hydrolysis ladder from the target RNA.

Consistent with the previous observation (Zamore et al., 2000), all target RNA cleavage sites were located within the region of identity to the dsRNA. The sense or the antisense target was only cleaved once by 39 bp dsRNA. Each cleavage site was located 10 nt from the 5' end of the region covered by the dsRNA (Figure 3B). The 52 bp dsRNA, which shares the same 5' end with the 39 bp dsRNA, produces the same cleavage site on the sense target, located 10 nt from the 5' end of the region of identity with the dsRNA, in addition to two weaker cleavage sites 23 and 24 nt downstream of the first site. The antisense target was only cleaved once, again 10 nt from the 5' end of the region covered by its respective dsRNA. Mapping of the cleavage sites for the 38 to 49 bp dsRNAs shown in Figure 1 showed that the first and predominant cleavage site was always located 7 to 10 nt downstream of the region covered by the dsRNA (data not

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shown). This suggests that the point of target RNA cleavage is determined by the end of the dsRNA and could imply that processing to 21-23 mers starts from the ends of the duplex.

5 Cleavage sites on sense and antisense target for the longer 111 bp dsRNA were much more frequent than anticipated and most of them appear in clusters separated by 20 to 23 nt (Figures 3A and 3B). As for the shorter dsRNAs, the first cleavage site on the sense target is 10 nt from the 5' end of the region spanned by the dsRNA, and the first cleavage site on the  
10 antisense target is located 9 nt from the 5' end of region covered by the dsRNA. It is unclear what causes this disordered cleavage, but one possibility could be that longer dsRNAs may not only get processed from the ends but also internally, or there are some specificity determinants for dsRNA processing which we do not yet understand. Some irregularities to  
15 the 21-23 nt spacing were also previously noted (Zamore et al., 2000). To better understand the molecular basis of dsRNA processing and target RNA recognition, we decided to analyze the sequences of the 21-23 nt fragments generated by processing of 39, 52, and 111 bp dsRNAs in the *Drosophila* lysate.

20

### **2.3 dsRNA is Processed to 21 and 22 nt RNAs by an RNase III-Like Mechanism**

In order to characterize the 21-23 nt RNA fragments we examined the 5' and 3' termini of the RNA fragments. Periodate oxidation of gel-purified 21-  
25 23 nt RNAs followed by  $\beta$ -elimination indicated the presence of a terminal 2' and 3' hydroxyl groups. The 21-23 mers were also responsive to alkaline phosphatase treatment indicating the presence of a 5' terminal phosphate group. The presence of 5' phosphate and 3' hydroxyl termini suggests that the dsRNA could be processed by an enzymatic activity  
30 similar to *E. coli* RNase III (for reviews, see (Dunn, 1982; Nicholson, 1999; Robertson, 1990; Robertson, 1982)).

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Directional cloning of 21-23 nt RNA fragments was performed by ligation of a 3' and 5' adapter oligonucleotide to the purified 21-23 mers using T4 RNA ligase. The ligation products were reverse transcribed, PCR-amplified, concatamerized, cloned, and sequenced. Over 220 short RNAs were  
5 sequenced from dsRNA processing reactions of the 39, 52 and 111 bp dsRNAs (Figure 4A). We found the following length distribution: 1% 18 nt, 5% 19 nt, 12% 20 nt, 45% 21 nt, 28% 22 nt, 6% 23 nt, and 2% 24 nt. Sequence analysis of the 5' terminal nucleotide of the processed fragments indicated that oligonucleotides with a 5' guanosine were underrepresented.  
10 This bias was most likely introduced by T4 RNA ligase which discriminates against 5' phosphorylated guanosine as donor oligonucleotide; no significant sequence bias was seen at the 3' end. Many of the ~21 nt fragments derived from the 3' ends of the sense or antisense strand of the duplexes include 3' nucleotides that are derived from untemplated addition of nu-  
15 cleotides during RNA synthesis using T7 RNA polymerase. Interestingly, a significant number of endogenous *Drosophila* ~21 nt RNAs were also cloned, some of them from LTR and non-LTR retrotransposons (data not shown). This is consistent with a possible role for RNAi in transposon silencing.

20

The ~21 nt RNAs appear in clustered groups (Figure 4A) which cover the entire dsRNA sequences. Apparently, the processing reaction cuts the dsRNA by leaving staggered 3' ends, another characteristic of RNase III cleavage. For the 39 bp dsRNA, two clusters of ~21 nt RNAs were found  
25 from each dsRNA-constituting strand including overhanging 3' ends, yet only one cleavage site was detected on the sense and antisense target (Figures 3A and 3B). If the ~21 nt fragments were present as single-stranded guide RNAs in a complex that mediates mRNA degradation, it could be assumed that at least two target cleavage sites exist, but this  
30 was not the case. This suggests that the ~21 nt RNAs may be present in double-stranded form in the endonuclease complex but that only one of the strands can be used for target RNA recognition and cleavage. The use of

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only one of the  $\sim 21$  nt strands for target cleavage may simply be determined by the orientation in which the  $\sim 21$  nt duplex is bound to the nuclease complex. This orientation is defined by the direction in which the original dsRNA was processed.

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The  $\sim 21$ mer clusters for the 52 bp and 111 bp dsRNA are less well defined when compared to the 39 bp dsRNA. The clusters are spread over regions of 25 to 30 nt most likely representing several distinct subpopulations of  $\sim 21$  nt duplexes and therefore guiding target cleavage at several nearby sites. These cleavage regions are still predominantly separated by 20 to 23 nt intervals. The rules determining how regular dsRNA can be processed to  $\sim 21$  nt fragments are not yet understood, but it was previously observed that the approx. 21-23 nt spacing of cleavage sites could be altered by a run of uridines (Zamore et al., 2000). The specificity of dsRNA cleavage by *E. coli* RNase III appears to be mainly controlled by antideterminants, i.e. excluding some specific base-pairs at given positions relative to the cleavage site (Zhang and Nicholson, 1997).

15

To test whether sugar-, base- or cap-modification were present in processed  $\sim 21$  nt RNA fragments, we incubated radiolabeled 505 bp Pp-luc dsRNA in lysate for 1 h, isolated the  $\sim 21$  nt products, and digested it with P1 or T2 nuclease to mononucleotides. The nucleotide mixture was then analyzed by 2D thin-layer chromatography (Figure 4B). None of the four natural ribonucleotides were modified as indicated by P1 or T2 digestion. We have previously analyzed adenosine to inosine conversion in the  $\sim 21$  nt fragments (after a 2 h incubation) and detected a small extent ( $<0.7\%$ ) deamination (Zamore et al., 2000); shorter incubation in lysate (1 h) reduced this inosine fraction to barely detectable levels. RNase T2, which cleaves 3' of the phosphodiester linkage, produced nucleoside 3'-phosphate and nucleoside 3',5'-diphosphate, thereby indicating the presence of a 5'-terminal monophosphate. All four nucleoside 3',5'-diphosphates were detected and suggest that the internucleotidic linkage was

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cleaved with little or no sequence-specificity. In summary, the ~21 nt fragments are unmodified and were generated from dsRNA such that 5'-monophosphates and 3'-hydroxyls were present at the 5'-end.

#### 5     **2.4 Synthetic 21 and 22 nt RNAs Mediate Target RNA Cleavage**

Analysis of the products of dsRNA processing indicated that the ~21 nt fragments are generated by a reaction with all the characteristics of an RNase III cleavage reaction (Dunn, 1982; Nicholson, 1999; Robertson, 1990; Robertson, 1982). RNase III makes two staggered cuts in both  
10 strands of the dsRNA, leaving a 3' overhang of about 2 nt. We chemically synthesized 21 and 22 nt RNAs, identical in sequence to some of the cloned ~21 nt fragments, and tested them for their ability to mediate target RNA degradation (Figures 5A and 5B). The 21 and 22 nt RNA duplexes were incubated at 100 nM concentrations in the lysate, a 10-fold  
15 higher concentrations than the 52 bp control dsRNA. Under these conditions, target RNA cleavage is readily detectable. Reducing the concentration of 21 and 22 nt duplexes from 100 to 10 nM does still cause target RNA cleavage. Increasing the duplex concentration from 100 nM to 1000 nM however does not further increase target cleavage, probably due to a  
20 limiting protein factor within the lysate.

In contrast to 29 or 30 bp dsRNAs that did not mediate RNAi, the 21 and 22 nt dsRNAs with overhanging 3' ends of 2 to 4 nt mediated efficient degradation of target RNA (duplexes 1, 3, 4, 6, Figures 5A and 5B). Blunt-  
25 ended 21 or 22 nt dsRNAs (duplexes 2, 5, and 7, Figures 5A and 5B) were reduced in their ability to degrade the target and indicate that overhanging 3' ends are critical for reconstitution of the RNA-protein nuclease complex. The single-stranded overhangs may be required for high affinity binding of the ~ 21 nt duplex to the protein components. A 5' terminal phosphate,  
30 although present after dsRNA processing, was not required to mediate target RNA cleavage and was absent from the short synthetic RNAs.

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The synthetic 21 and 22 nt duplexes guided cleavage of sense as well as antisense targets within the region covered by the short duplex. This is an important result considering that a 39 bp dsRNA, which forms two pairs of clusters of ~21 nt fragments (Fig. 2), cleaved sense or antisense target only once and not twice. We interpret this result by suggesting that only one of two strands present in the ~21 nt duplex is able to guide target RNA cleavage and that the orientation of the ~21 nt duplex in the nuclease complex is determined by the initial direction of dsRNA processing. The presentation of an already perfectly processed ~21 nt duplex to the in vitro system however does allow formation of the active sequence-specific nuclease complex with two possible orientations of the symmetric RNA duplex. This results in cleavage of sense as well as antisense target within the region of identity with the 21 nt RNA duplex.

The target cleavage site is located 11 or 12 nt downstream of the first nucleotide that is complementary to the 21 or 22 nt guide sequence, i.e. the cleavage site is near center of the region covered by the 21 or 22 nt RNAs (Figures 4A and 4B). Displacing the sense strand of a 22 nt duplex by two nucleotides (compare duplexes 1 and 3 in Figure 5A) displaced the cleavage site of only the antisense target by two nucleotides. Displacing both sense and antisense strand by two nucleotides shifted both cleavage sites by two nucleotides (compare duplexes 1 and 4). We predict that it will be possible to design a pair of 21 or 22 nt RNAs to cleave a target RNA at almost any given position.

The specificity of target RNA cleavage guided by 21 and 22 nt RNAs appears exquisite as no aberrant cleavage sites are detected (Figure 5B). It should however be noted, that the nucleotides present in the 3' overhang of the 21 and 22 nt RNA duplex may contribute less to substrate recognition than the nucleotides near the cleavage site. This is based on the observation that the 3' most nucleotide in the 3' overhang of the active duplexes 1 or 3 (Figure 5A) is not complementary to the target. A detailed

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analysis of the specificity of RNAi can now be readily undertaken using synthetic 21 and 22 nt RNAs.

Based on the evidence that synthetic 21 and 22 nt RNAs with overhanging 3' ends mediate RNA interference, we propose to name the ~21 nt RNAs "short interfering RNAs" or siRNAs and the respective RNA-protein complex a "small interfering ribonucleoprotein particle" or siRNP.

### 2.5 3' Overhangs of 20 nt on short dsRNAs inhibit RNAi

We have shown that short blunt-ended dsRNAs appear to be processed from the ends of the dsRNA. During our study of the length dependence of dsRNA in RNAi, we have also analyzed dsRNAs with 17 to 20 nt overhanging 3' ends and found to our surprise that they were less potent than blunt-ended dsRNAs. The inhibitory effect of long 3' ends was particularly pronounced for dsRNAs up to 100 bp but was less dramatic for longer dsRNAs. The effect was not due to imperfect dsRNA formation based on native gel analysis (data not shown). We tested if the inhibitory effect of long overhanging 3' ends could be used as a tool to direct dsRNA processing to only one of the two ends of a short RNA duplex.

We synthesized four combinations of the 52 bp model dsRNA, blunt-ended, 3' extension on only the sense strand, 3'-extension on only the antisense strand, and double 3' extension on both strands, and mapped the target RNA cleavage sites after incubation in lysate (Figures 6A and 6B). The first and predominant cleavage site of the sense target was lost when the 3' end of the antisense strand of the duplex was extended, and vice versa, the strong cleavage site of the antisense target was lost when the 3' end of sense strand of the duplex was extended. 3' Extensions on both strands rendered the 52 bp dsRNA virtually inactive. One explanation for the dsRNA inactivation by ~20 nt 3' extensions could be the association of single-stranded RNA-binding proteins which could interfere with the association of one of the dsRNA-processing factors at this end. This result

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is also consistent with our model where only one of the strands of the siRNA duplex in the assembled siRNP is able to guide target RNA cleavage. The orientation of the strand that guides RNA cleavage is defined by the direction of the dsRNA processing reaction. It is likely that the presence of 3' staggered ends may facilitate the assembly of the processing complex. A block at the 3' end of the sense strand will only permit dsRNA processing from the opposing 3' end of the antisense strand. This in turn generates siRNP complexes in which only the antisense strand of the siRNA duplex is able to guide sense target RNA cleavage. The same is true for the reciprocal situation.

The less pronounced inhibitory effect of long 3' extensions in the case of longer dsRNAs ( $\geq 500$  bp, data not shown) suggests to us that long dsRNAs may also contain internal dsRNA-processing signals or may get processed cooperatively due to the association of multiple cleavage factors.

## 2.6 A Model for dsRNA-Directed mRNA Cleavage

The new biochemical data update the model for how dsRNA targets mRNA for destruction (Figure 7). Double-stranded RNA is first processed to short RNA duplexes of predominantly 21 and 22 nt in length and with staggered 3' ends similar to an RNase III-like reaction (Dunn, 1982; Nicholson, 1999; Robertson, 1982). Based on the 21-23 nt length of the processed RNA fragments it has already been speculated that an RNase III-like activity may be involved in RNAi (Bass, 2000). This hypothesis is further supported by the presence of 5' phosphates and 3' hydroxyls at the termini of the siRNAs as observed in RNase III reaction products (Dunn, 1982; Nicholson, 1999). Bacterial RNase III and the eukaryotic homologs Rnt1p in *S. cerevisiae* and Pac1p in *S. pombe* have been shown to function in processing of ribosomal RNA as well as snRNA and snoRNAs (see for example Chanfreau et al., 2000).

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Little is known about the biochemistry of RNase III homologs from plants, animals or human. Two families of RNase III enzymes have been identified predominantly by database-guided sequence analysis or cloning of cDNAs. The first RNase III family is represented by the 1327 amino acid long D. melanogaster protein drosha (Acc. AF116572). The C-terminus is composed of two RNase III and one dsRNA-binding domain and the N-terminus is of unknown function. Close homologs are also found in C. elegans (Acc. AF160248) and human (Acc. AF189011) (Filippov et al., 2000; Wu et al., 2000). The drosha-like human RNase III was recently cloned and characterized (Wu et al., 2000). The gene is ubiquitously expressed in human tissues and cell lines, and the protein is localized in the nucleus and the nucleolus of the cell. Based on results inferred from antisense inhibition studies, a role of this protein for rRNA processing was suggested. The second class is represented by the C. elegans gene K12H4.8 (Acc. S44849) coding for a 1822 amino acid long protein. This protein has an N-terminal RNA helicase motif which is followed by 2 RNase III catalytic domains and a dsRNA-binding motif, similar to the drosha RNase III family. There are close homologs in S. pombe (Acc. Q09884), A. thaliana (Acc. AF187317), D. melanogaster (Acc. AE003740), and human (Acc. AB028449) (Filippov et al., 2000; Jacobsen et al., 1999; Matsuda et al., 2000). Possibly the K12H4.8 RNase III/helicase is the likely candidate to be involved in RNAi.

Genetic screens in C. elegans identified rde-1 and rde-4 as essential for activation of RNAi without an effect on transposon mobilization or co-suppression (Dernburg et al., 2000; Grishok et al., 2000; Ketting and Plasterk, 2000; Tabara et al., 1999). This led to the hypothesis that these genes are important for dsRNA processing but are not involved in mRNA target degradation. The function of both genes is as yet unknown, the rde-1 gene product is a member of a family of proteins similar to the rabbit protein eIF2C (Tabara et al., 1999), and the sequence of rde-4 has not yet

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been described. Future biochemical characterization of these proteins should reveal their molecular function.

Processing to the siRNA duplexes appears to start from the ends of both  
5 blunt-ended dsRNAs or dsRNAs with short (1-5 nt) 3' overhangs, and  
proceeds in approximately 21-23 nt steps. Long (~20 nt) 3' staggered  
ends on short dsRNAs suppress RNAi, possibly through interaction with  
single-stranded RNA-binding proteins. The suppression of RNAi by single-  
stranded regions flanking short dsRNA and the lack of siRNA formation  
10 from short 30 bp dsRNAs may explain why structured regions frequently  
encountered in mRNAs do not lead to activation of RNAi.

Without wishing to be bound by theory, we presume that the dsRNA-  
processing proteins or a subset of these remain associated with the siRNA  
15 duplex after the processing reaction. The orientation of the siRNA duplex  
relative to these proteins determines which of the two complementary  
strands functions in guiding target RNA degradation. Chemically syn-  
thesized siRNA duplexes guide cleavage of sense as well as antisense  
target RNA as they are able to associate with the protein components in  
20 either of the two possible orientation.

The remarkable finding that synthetic 21 and 22 nt siRNA duplexes can be  
used for efficient mRNA degradation provides new tools for sequence-  
specific regulation of gene expression in functional genomics as well as  
25 biomedical studies. The siRNAs may be effective in mammalian systems  
where long dsRNAs cannot be used due to the activation of the PKR  
response (Clemens, 1997). As such, the siRNA duplexes represent a new  
alternative to antisense or ribozyme therapeutics.

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## Claims

- 5 1. Isolated double-stranded RNA molecule, wherein each RNA strand has a length from 19-23 nucleotides, wherein said RNA molecule is capable of target-specific nucleic acid modifications.
- 10 2. The RNA molecule of claim 1 wherein at least one strand has a 3'-overhang from 1-5 nucleotides.
3. The RNA molecule of claim 1 or 2 capable of target-specific RNA interference and/or DNA methylation.
- 15 4. The RNA molecule of any one of claims 1-3, wherein each strand has a length from 20-22 nucleotides.
5. The RNA molecule of any one of claims 2-4, wherein the 3'-overhang is from 1-3 nucleotides.
- 20 6. The RNA molecule of any one of claims 2-5, wherein the 3'-overhang is stabilized against degradation.
7. The RNA molecule of any one of claims 1-6, which contains at least one modified nucleotide analogue.
- 25 8. The RNA molecule of claim 7, wherein the modified nucleotide analogue is selected from sugar- or backbone modified ribonucleotides.
- 30 9. The RNA molecule according to claim 7 or 8, wherein the nucleotide analogue is a sugar-modified ribonucleotide, wherein the 2'-OH group is replaced by a group selected from H, OR, R, halo, SH, SR<sup>1</sup>,

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NH<sub>2</sub>, NHR, NR<sub>2</sub> or CN, wherein R is C<sub>1</sub>-C<sub>6</sub> alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I.

- 5      10.    The RNA molecule of claim 7 or 8, wherein the nucleotide analogue is a backbone-modified ribonucleotide containing a phosphothioate group.
- 10      11.    The RNA molecule of any one of claims 1-10, which has a sequence having an identity of at least 50 percent to a predetermined mRNA target molecule.
12.    The RNA molecule of claim 11, wherein the identity is at least 70 percent.
- 15      13.    A method of preparing a double-stranded RNA molecule of any one of claims 1-12 comprising the steps:
- 20      (a)    synthesizing two RNA strands each having a length from 19-23 nucleotides, wherein said RNA strands are capable of forming a double-stranded RNA molecule,
- 25      (b)    combining the synthesized RNA strands under conditions, wherein a double-stranded RNA molecule is formed, which is capable of target-specific nucleic acid modifications.
14.    The method of claim 13, wherein the RNA strands are chemically synthesized.
- 30      15.    The method of claim 13, wherein the RNA strands are enzymatically synthesized.

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16. A method of mediating target-specific nucleic acid modifications in a cell or an organism comprising the steps:
- 5 (a) contacting said cell or organism with the double-stranded RNA molecule of any one of claims 1-12 and
- (b) mediating a target-specific nucleic acid modification effected by the double-stranded RNA towards a target nucleic acid having a sequence portion substantially corresponding to the double-stranded RNA.
- 10
17. The method of claim 16, wherein the nucleic acid modification is RNA interference and/or DNA methylation.
- 15 18. Use of the method of claim 16 or 17 for determining the function of a gene in a cell or an organism.
19. Use of the method of claim 16 or 17 for modulating the function of a gene in a cell or an organism.
- 20 20. The use of claim 16 or 17, wherein the gene is associated with a pathological condition.
21. The use of claim 20, wherein the gene is a pathogen-associated gene.
- 25 22. The use of claim 21, wherein the gene is a viral gene.
23. The use of claim 20, wherein the gene is a tumor-associated gene.
- 30 24. The use of claim 20, wherein the gene is an autoimmune disease-associated gene.

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25. Pharmaceutical composition containing as an active agent at least one double-stranded RNA molecule of any one of claims 1-12 and a pharmaceutical carrier.

5 26. The composition of claim 25 for diagnostic applications.

27. The composition of claim 25 for therapeutic applications.

10



**Abstract**

Double-stranded RNA (dsRNA) induces sequence-specific post-transcriptional gene silencing in many organisms by a process known as RNA interference (RNAi). Using a *Drosophila* in vitro system, we demonstrate that 19-23 nt short RNA fragments are the sequence-specific mediators of RNAi. The short interfering RNAs (siRNAs) are generated by an RNase III-like processing reaction from long dsRNA. Chemically synthesized siRNA duplexes with overhanging 3' ends mediate efficient target RNA cleavage in the lysate, and the cleavage site is located near the center of the region spanned by the guiding siRNA. Furthermore, we provide evidence that the direction of dsRNA processing determines whether sense or antisense target RNA can be cleaved by the produced siRNP complex.

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FIGURE 1A

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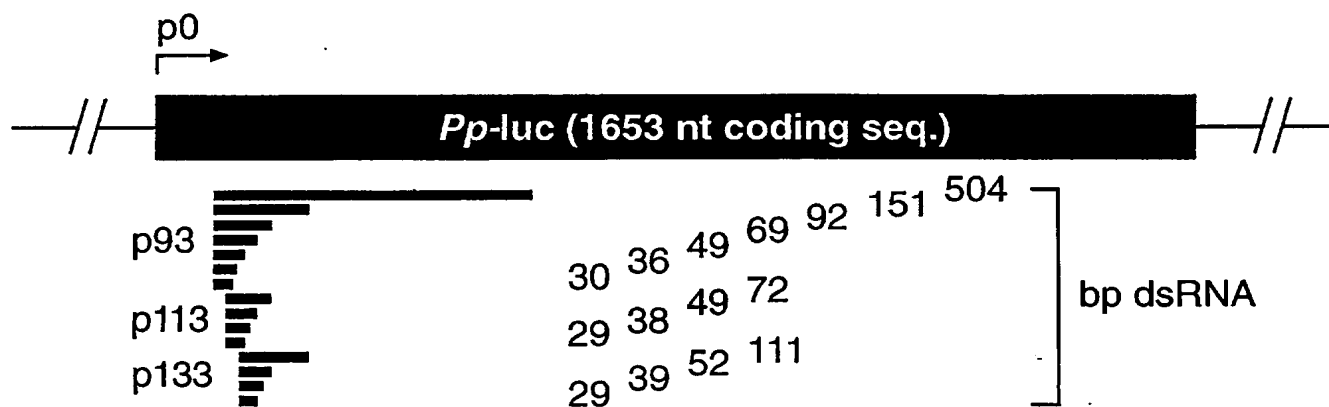


FIGURE 1B

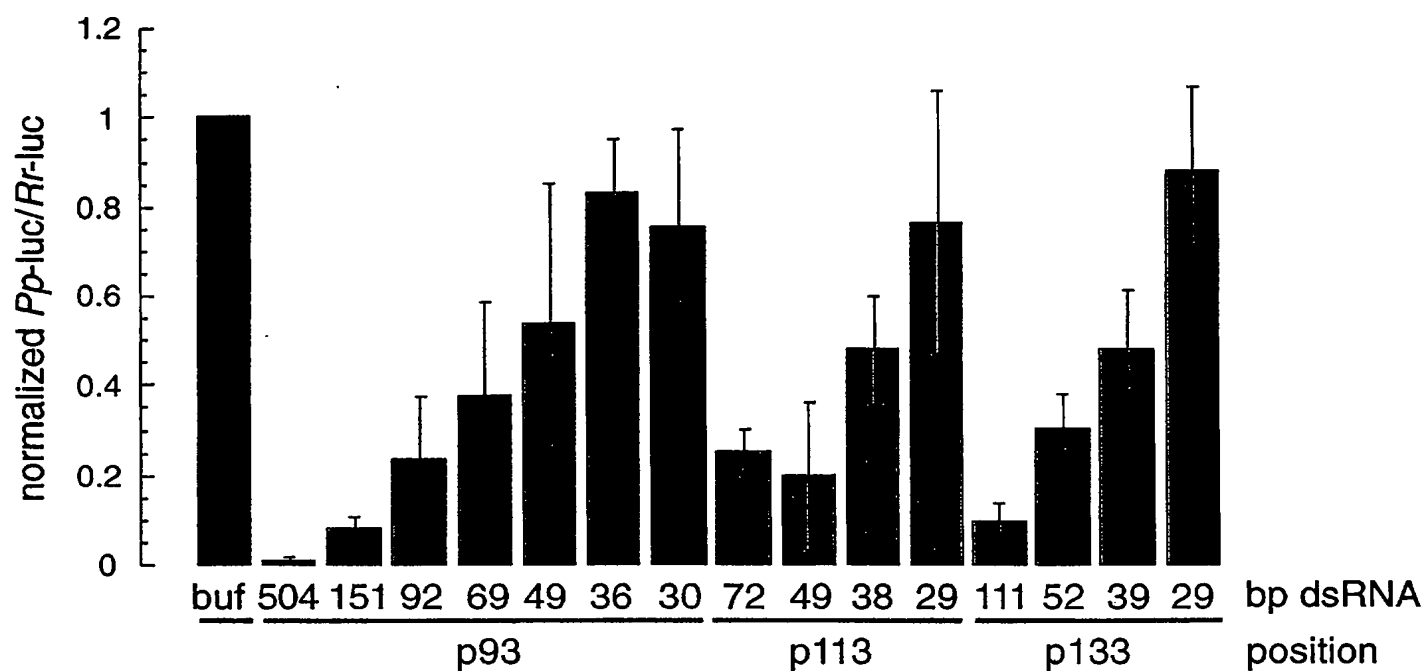


FIGURE 2

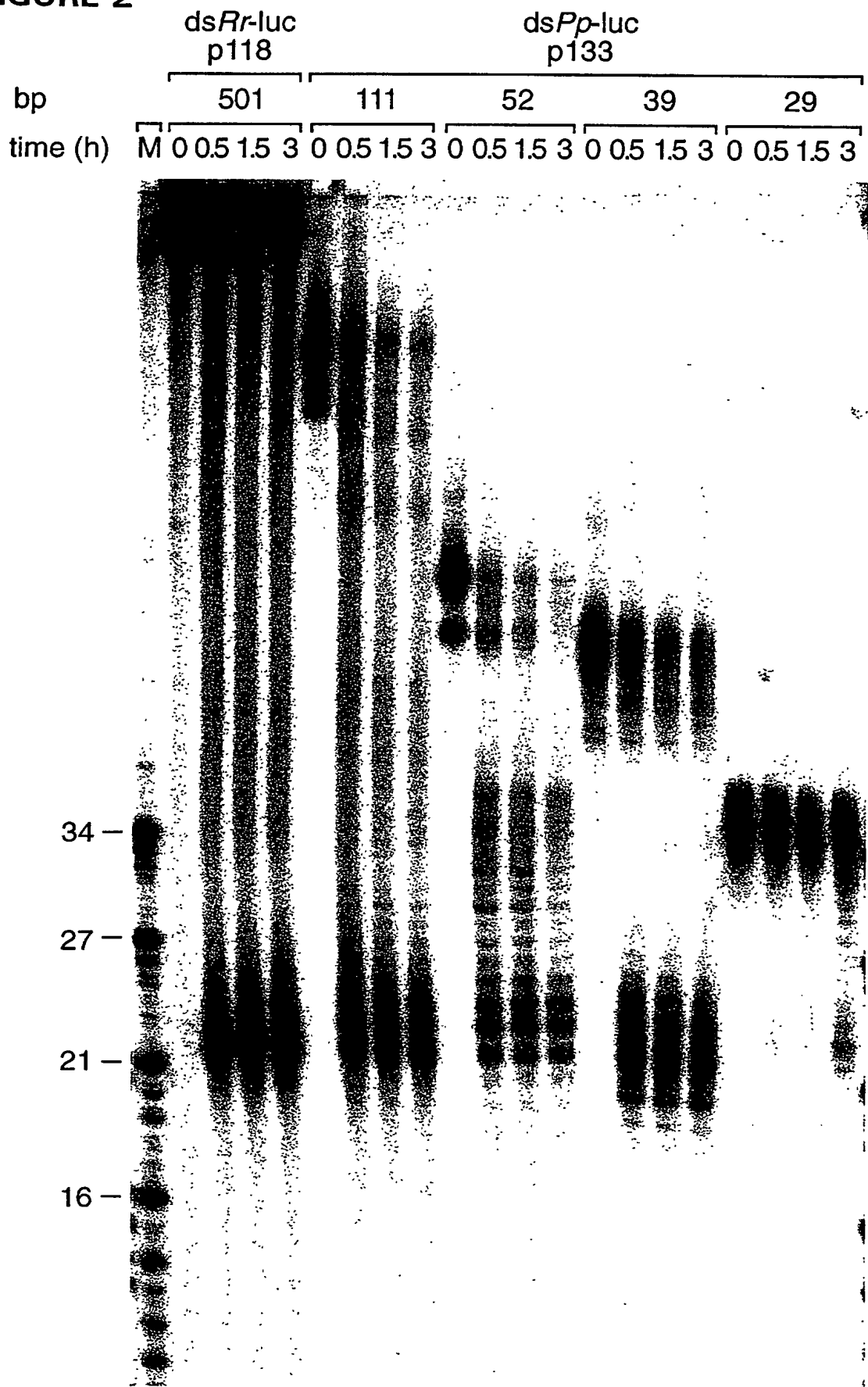


FIGURE 3A

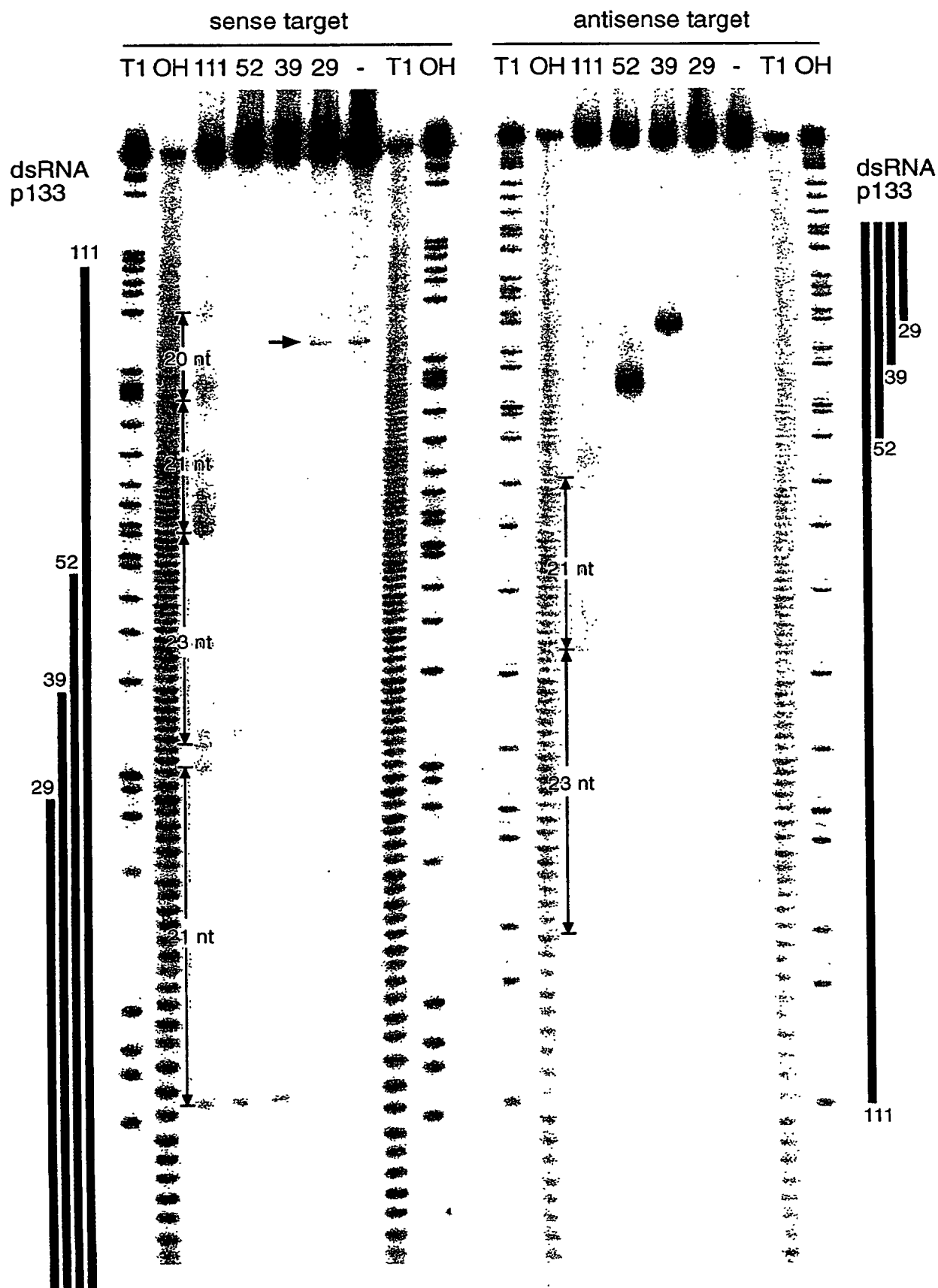
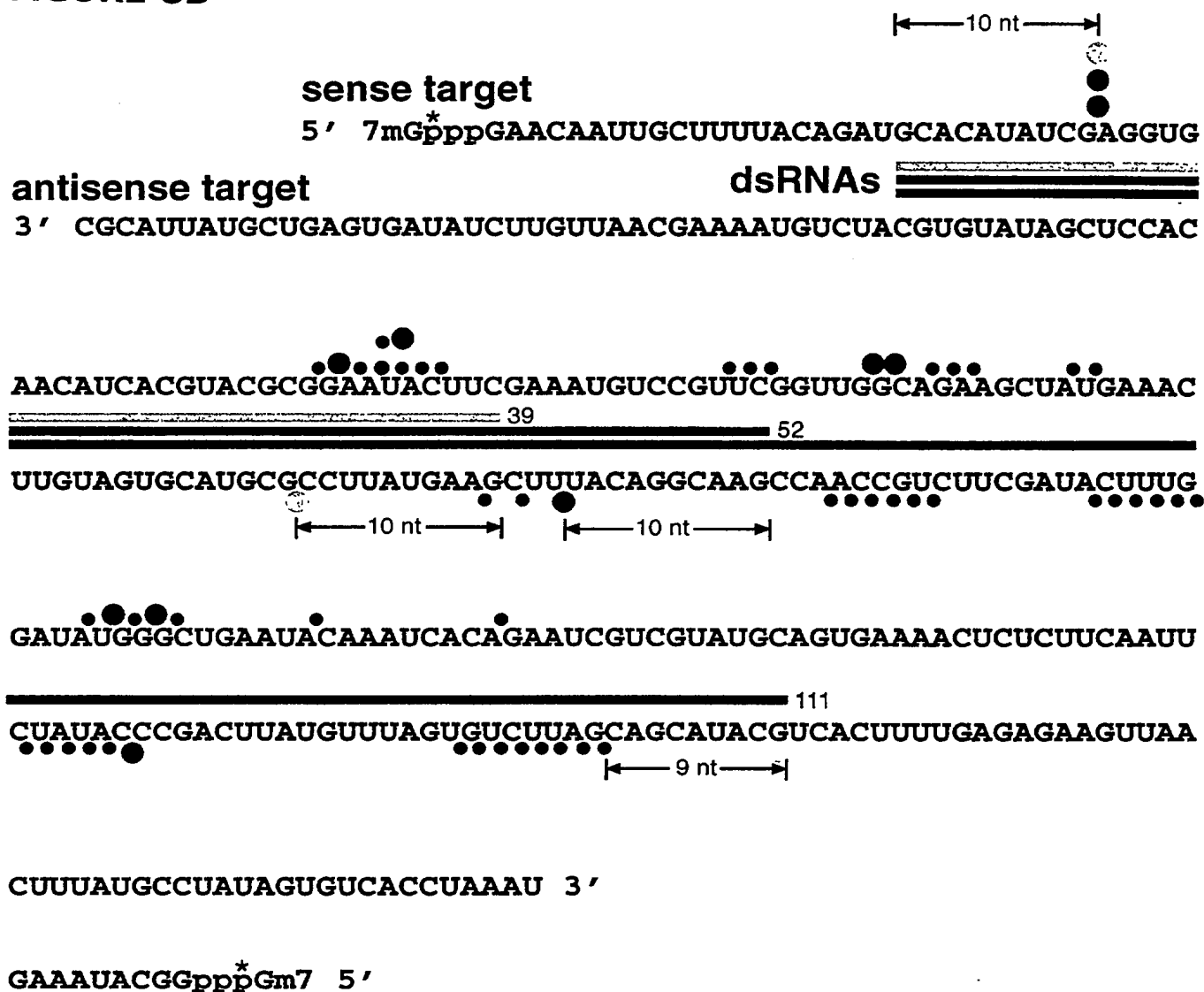


FIGURE 3B



## FIGURE 4A

39 bp ds*Pp*-luc p133


5' GCACAUAUCGAGGUGAACAUCACGUACGCGGAAUACUUC  
3' CGUGUAUAGCUCCACUUGUAGUGCAUGCGCCUUAUGAAG

52 bp ds*Pp*-luc p133


5' GCACAUAUCGAGGUGAACAUCACGUACGCGGAAUACUUCGAAAUGUCCGUUC  
3' CGUGUAUAGCUCCACUUGUAGUGCAUGCGCCUUAUGAAGCUUUACAGGCAAG

111 bp ds*Pp*-luc p133


5' GCACAUAUCGAGGUGAACAUCACGUACGCGGAAUACUUCGAAAUGUCCGUUCGGU  
3' CGUGUAUAGCUCCACUUGUAGUGCAUGCGCCUUAUGAAGCUUUACAGGCAAGCCA



UGGCAGAAGCUAUGAAACGAUAUGGGCUGAAUACAAUACAGAAUCGUCGUAUGC  
ACCGUCUUCGAUAUUCUUGCUAUACCCGACUUAUGUUUAGUGUCUUAAGCAGCAUACG

FIGURE 4B

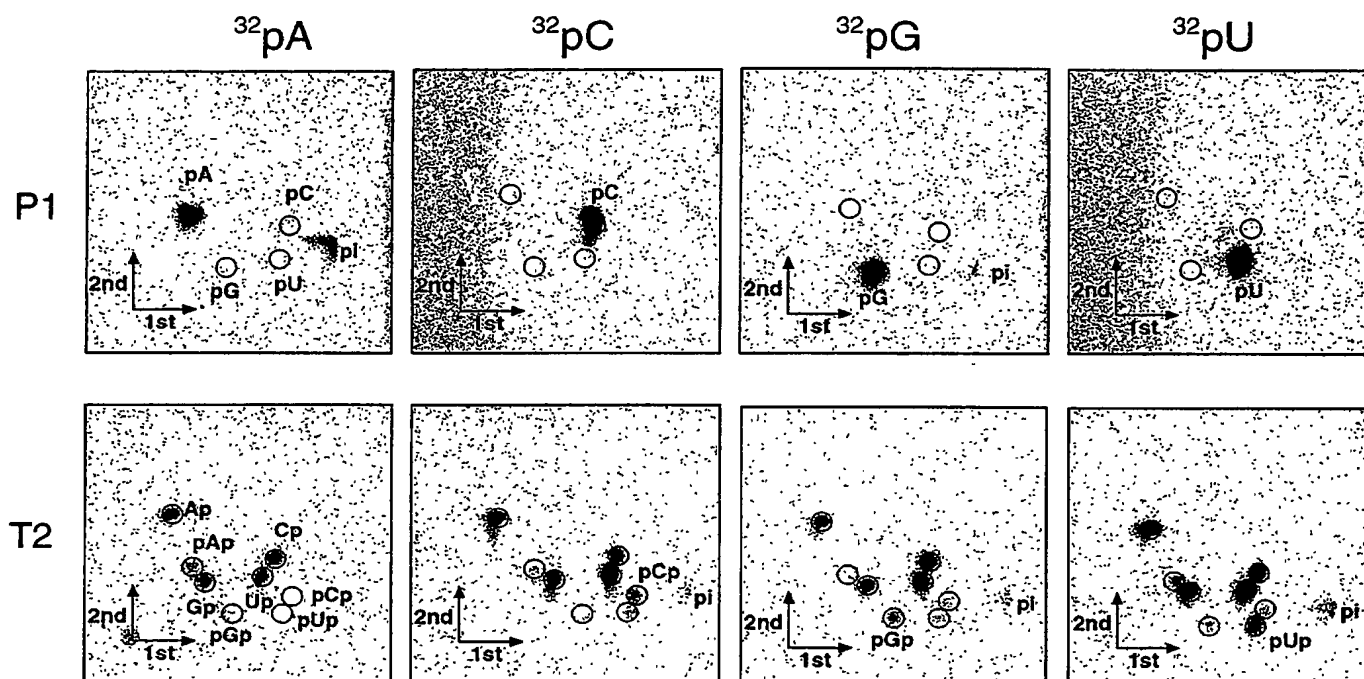


FIGURE 5A

siRNAs/  
dsRNA

ds52  
(control)

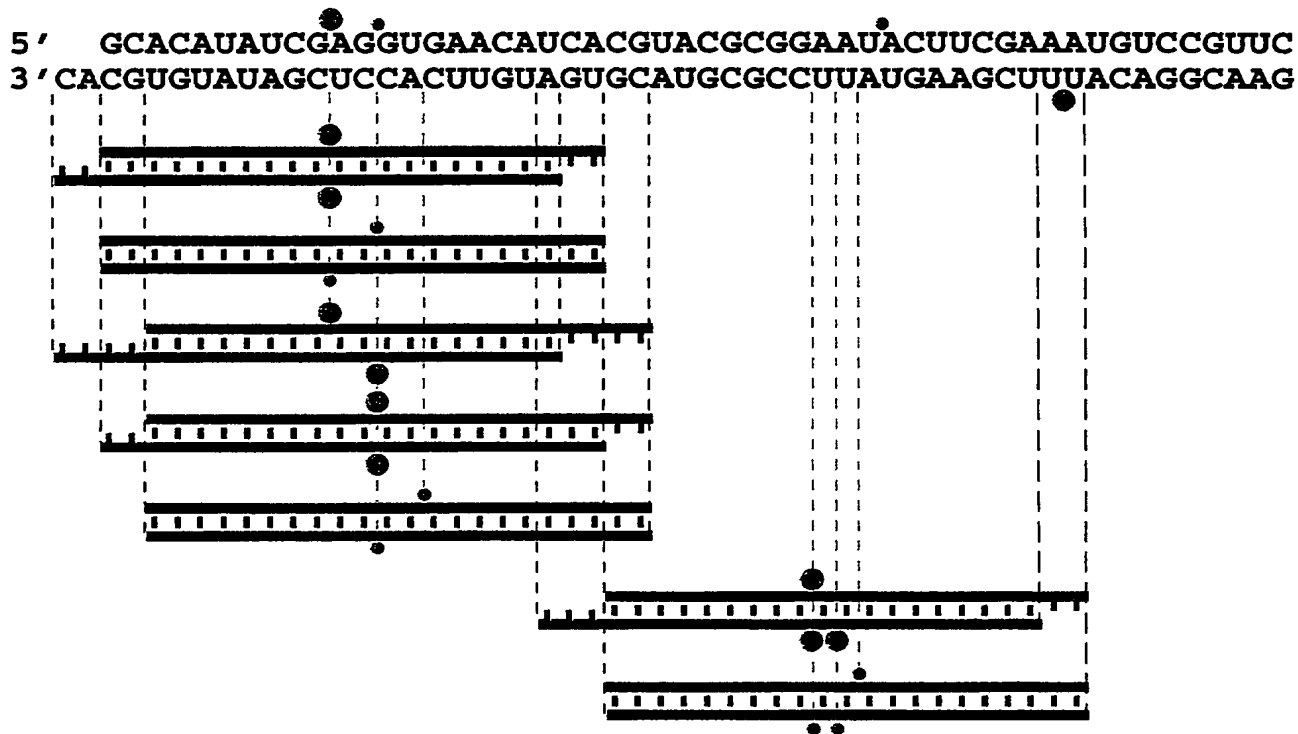




FIGURE 5B

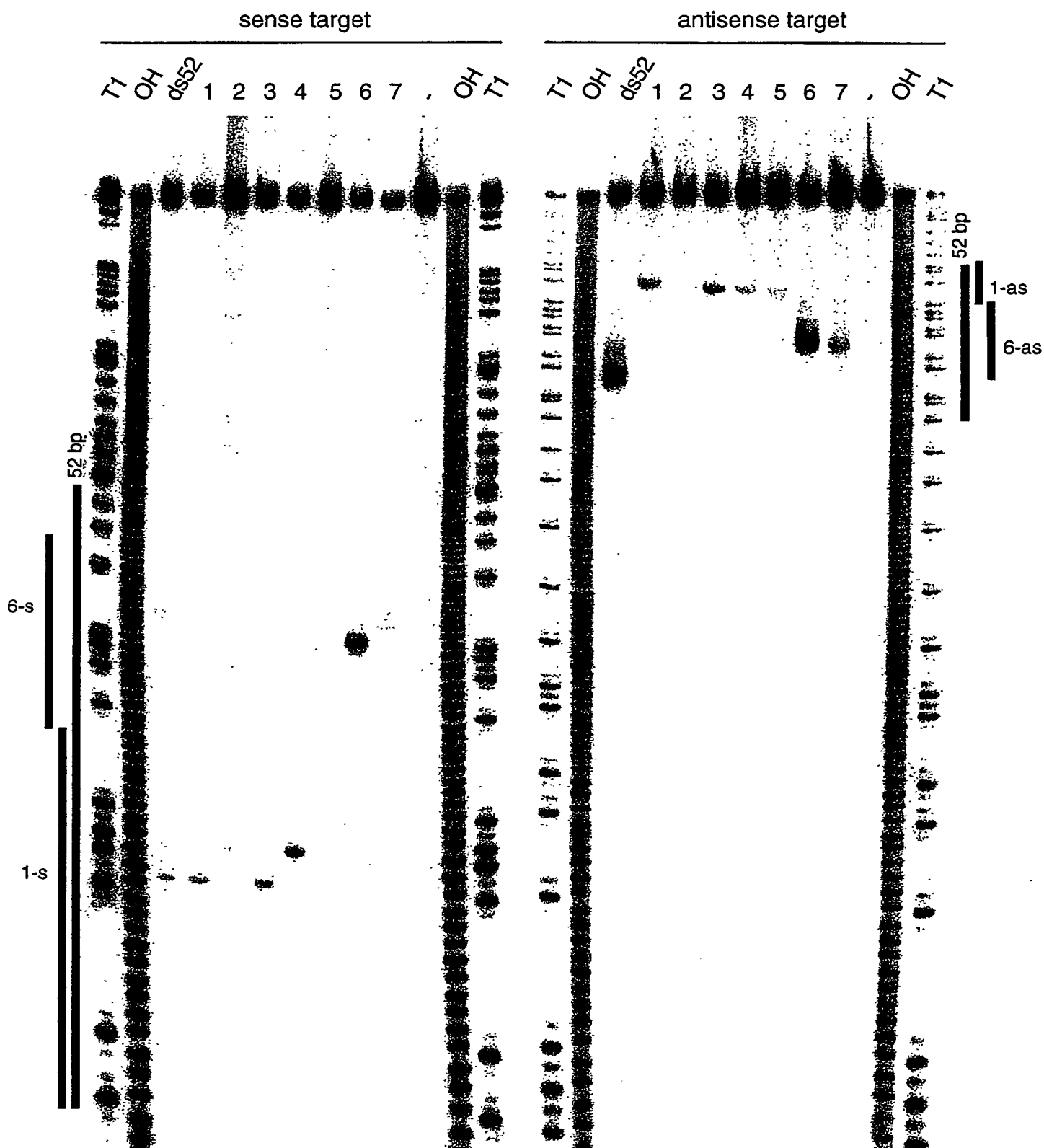


FIGURE 6A

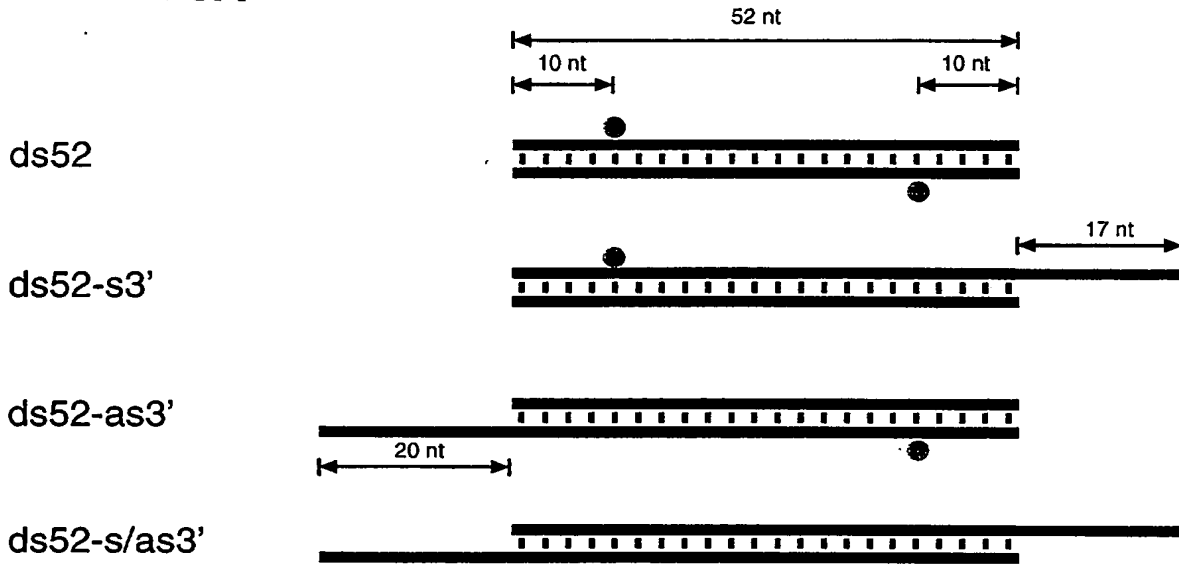


FIGURE 6B

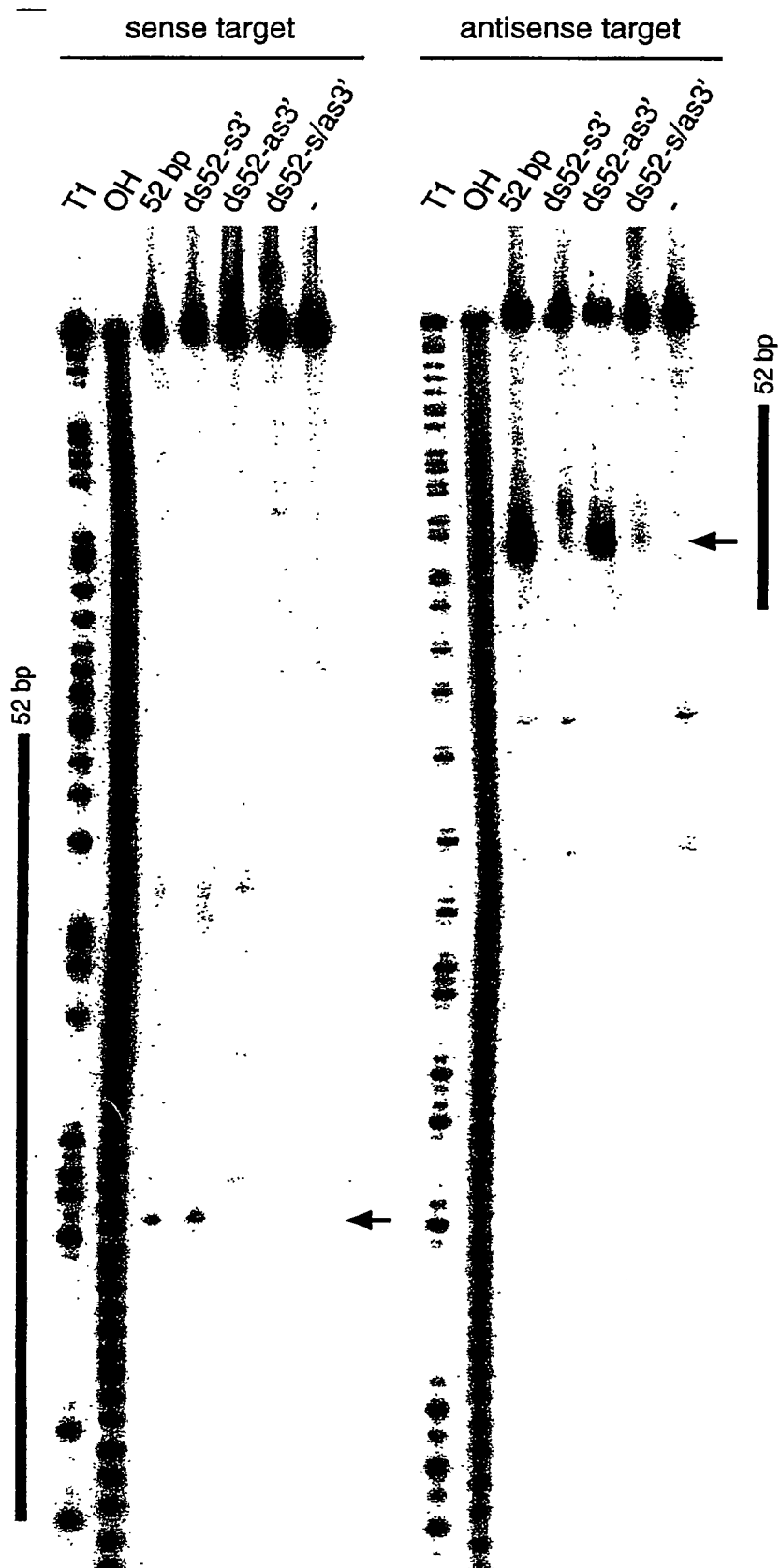


FIGURE 7

